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Application of IL-5 ELISPOT assays to quantification of antigen-specific T helper responses

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Abstract

To be able to determine the frequencies of helper-type T lymphocyte (Th) precursors specific for viral or tumor-associated antigens, an ELISPOT assay for IL-5 production was developed. The assay reproducibility was first determined using fresh or cryopreserved peripheral blood mononuclear cells (PBMC) obtained from six normal donors and tested for IL-5 production after 48 h stimulation with phytohemagglutinin (PHA). Both inter-assay (within-subject CV = 1.6%) and intra-assay (within-subject CV = 0.8%) variabilities were found to be acceptable, and the frequencies of IL-5 secreting cells were comparable in cryopreserved and fresh PBMC of the same donors. The presence and frequency of Th precursor cells to viral capsid L1 protein (viral-like particles, VLP-L1) of human papillomavirus type 16 (HPV-16) in PBMC obtained from seven non-immunized donors and two volunteers immunized with VLP-L1 were then evaluated. Using autologous dendritic cells as antigen-presenting cells (APC) for VLP-L1 in direct 48-h ELISPOT assays, the mean frequency of IL-5 secreting CD4+ T cells was found to be $< 1/10^5$ (negative) in normal donors but was 1/2, 436 and 1/3678 in the two volunteers immunized with VLP-L1. The assay is applicable to monitoring of the frequency of antigen-specific T cells in the peripheral circulation of individuals immunized with HPV-derived antigens. To test the assay utility in the assessment of Th tumor-reactive lymphocytes, we also used it to determine the frequency of the wild-type sequence (wt) $p53_{22-36}$ peptide-specific, HLA-DR4-restricted T cells in the bulk CD4+ T cell line. This frequency was 1/33. The ELISPOT assay for IL-5 production can be reliably used to measure Th-type responses in a variety of experimental settings.

Keywords: ELISPOT assay; IL-5; T-cell frequency; Helper T cells

1. Introduction

Mosmann et al. (1986) reported that the two subsets of helper T cells (Th), the Th1 and Th2, can be distinguished by their cytokine production profile (Mosmann et al., 1986). The Th1 cells secrete IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor-

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alpha (TNF-α), and the Th2 cells produce IL-4, IL-5, IL-6, IL-10 and/or IL-13 (Lucey et al., 1996). The two subsets of T helper cells also mediate different types of immune responses, with Th1 cells involved in the helper activity for cell-mediated responses, and Th2 cells engaged in the process of B-cell differentiation and antibody production. Initially, activated B cells produce IgM antibodies. The subsequent Ig class switching is dependent on the presence of cytokines secreted by Th cells. For example, IgG1 production is strictly dependent on IFN-γ production by Th1 cells (Coffman, 1993; Snapper and Paul, 1987). Maturation of cytotoxic T cells is dependent on the presence of Th1 cytokines as well. Immunization/vaccination of humans with epitopes derived from tumors or viral antigens is expected to induce both cytotoxic and helper responses specific for the immunogen. The former are mediated by CD8+ T cells, and the frequency of such epitope-specific effector T cells in the peripheral circulation can be determined using ELISPOT assays for IFN-γ production (Asai et al., 2000).

In general, the goal of anti-cancer or anti-viral vaccines is to induce a robust immune response to the immunogenic antigens, with contributions from both $CD4^+$ and $CD8^+$ as well as B cells (Sahin et al., 1995). Deficiencies in helper T cell responses have been observed in some patients with cancer or chronic viral infections, and it is expected that vaccination might restore normal helper functions in such individuals. Furthermore, prophylactic anti-viral vaccines, such as an HPV-16 VLP-L1-based vaccine for women at high risk of the development of cervical cancer, are being evaluated in hope of preventing cancer (Harro et al., 2001; Schiller and Lowy, 1996). In this setting, the demonstration of increased Th-cell responses to VLP might be particularly important. Thus, the monitoring of patients with cancer or infectious diseases who receive therapeutic or prophylactic vaccines, should include an assessment of changes in the frequency of circulating Th1-type and Th2-type cells in order to comprehensively evaluate the magnitude of the T-cell response to vaccines and, perhaps, to relate this response to clinical outcome. Furthermore, the correlation of Th2-type helper responses to humoral immunity also merits attention. Indeed, in some patients with cancer, antibody responses have been found to increase in parallel with cytotoxic responses to specific tumor antigens, e.g., NY-ESO-1 and HER-2/neu, following vaccination with the respective peptides (Zeng et al., 2001; Disis et al., 1994). The p53 antigen, which has been considered as a candidate for vaccination, is known to be able to induce humoral responses, with a high titer of anti-p53 antibodies detected in a subset of patients with cancer (Soussi, 2000). Similarly, in viral infections, for example, in hepatitis B and C, not only the presence of anti-viral antibodies in the serum, but also evidence for T-cell responses and for production of Th1-type and Th2-type cytokines has been demonstrated within the liver in chronic infections (Bertoletti et al., 1997).

In this manuscript, we describe the development, pre-clinical assessment, and application of the IL-5 ELISPOT assay for measuring the frequency of individual T cells which secrete IL-5 to monitoring of individuals vaccinated with HPV and to assessment of tumor antigen-specific $CD4^+$ T cells.

2. Materials and methods

2.1. Cytokines and antibodies

Cytokines used in this study were obtained from the following sources: interleukin-4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) from Schering Plough (Kennilworth, NJ); interleukin-1 β (IL-1 β) from National Cancer Institute, Biological Resources Branch, Frederick, MD; tumor necrosis factor α (TNF-α) from Knoll Pharmaceuticals (Whippany, NJ); interleukin-6 (IL-6) from Sandoz (Basel, Switzerland); interleukin-2 (IL-2) from Chiron-Cetus (Emeryville, CA); and Interleukin-7 (IL-7) from R&D Systems (Rockville, MD). The blocking antibodies, anti-MHC class I mAbs (HB95; W6.32) and anti-MHC class II (DR) mAbs (L243) were provided by Dr. Albert DeLeo (University of Pittsburgh Cancer Institute). FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and PerCP-conjugated anti-CD3 were all purchased from Becton Dickinson (Mountain View, CA).

2.2. Synthetic peptides and proteins

The following peptides were synthesized by the standard FMOC methodology: $p53_{22-36}$ (LWKLL PENNVLSPLP) and irrelevant $p53_{192-206}$ (QHLIR-

VEGNLRVEYL). Each peptide was purified by reverse phase HPLC, and the identity of each amino acid sequence was confirmed by mass spectrometry. The HPV16-L1 VLPs (Viral Like Particles) were obtained from the National Cancer Institute. These particles were composed only of the L1 protein, the major capsid protein of HPV which has the capacity to self-assemble into VLP when expressed in SF-9 insect cells infected with baculovirus (Novavax, Rockville, MD). The VLPs were dissolved in PBS at 0.757 mg/ml and stored at − 80 °C. The lysate of SF9 cells infected with baculovirus (Novavax) diluted in PBS to the concentration of 2.1 mg/ml was used as control. The lysate was titrated into medium and tested at different concentrations.

2.3. Peripheral blood mononuclear cells (PBMC)

Venous blood was obtained from six normal volunteers at several different time points over a year and collected into heparinized tubes. PBMC were isolated by Ficoll–Hypaque gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). PBMC recovered from the interface were washed, counted in a Trypan blue dye, and either immediately used for ELISPOT assays or cryopreserved in liquid N_2 for subsequent studies. Prior to use, cryopreserved samples were rapidly thawed. The recovered cells were washed, counted in a Trypan blue dye, and used for ELISPOT assays.

PBMC were also obtained by leukapheresis from two donors vaccinated with the HPV16 VLP-L1 experimental vaccine. Both donors received three doses of 50 μg of HPV-16-VLP-L1 without adjuvant. Leukapheresis was performed several months after the last vaccination. The leukapheresis product was delivered to the laboratory, PBMC were harvested on Ficoll–Hypaque, washed, counted, resuspended in the freezing medium, and cryopreserved in cryovials in liquid N_2, each vial containing from 15 to 20×10^6 cells. Prior to their use, the cells were thawed, washed four times, counted in a Trypan blue dye and either used directly for ELISPOT assays or cultured in the presence of antigen-presenting cells (APC). In some cases, PBMC were separated into $CD8^+$ or $CD4^+$ fractions, using positive selection with immunobeads according to the manufacturer's recommendations (MACS MicroBeads; Miltenyi Biotech, Auburn, CA). The purity of positively selected cells was typically 90%.

2.4. Flow cytometry

The purity of $CD4^+$ and CD8+ T-cell fractions after separation was evaluated by flow cytometry on FACScan (Becton Dickinson) after staining with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and PerCP-conjugated anti-CD3 Abs. Isotype Ig controls were used in all assays. The data were analysed using Lysis II software (Becton Dickinson).

2.5. Dendritic cell (DC) generation

DC were generated from cryopreserved PBMC which were thawed and suspended in serum-free AIM-V medium (Gibco/Life-Technologies, Grand Island, NY) at a cell density of 10^7/ml in T75 flasks (Falcon®, Becton Dickinson, Franklin Lakes, NJ). After 1 h of incubation at 37 °C, non-adherent cells were removed by three cycles of gentle washing with warm RPMI-medium (Gibco/Life-Technologies). The remaining adherent PBMC were resuspended in AIM-V medium supplemented with IL-4 (1000 IU/ml) and GM-CSF (1000 IU/ml) and cultured in an atmosphere of 5% CO_2 in air at 37 °C for 6 days. DC were harvested on day 7 and pulsed with HPV16-L1 VLP added at the concentration of 1 μg/ml. DC not pulsed with VLP or pulsed with an irrelevant antigen (ovalbumin at 5 μg/ml or baculovirus at 1 μg/ml) were used as negative controls. After 4 h of incubation in AIM-V medium, a cytokine cocktail (10 ng/ml of IL-1 β, 1000 U/ml of IL-6, 10 ng/ml of TNF-α) was added in order to mature DC in overnight cultures, as previously described (Jonuleit et al., 1997).

2.6. In vitro sensitization (IVS)

PBMC used as responding cells were thawed and resuspended at the final concentration of 5×10^5/ml in wells of a 24-well plate. Matured DC pulsed with the antigen were added to lymphocytes (L) to obtain a L:DC ratio of 10:1, and the cells were cultured in RPMI medium supplemented with 10% (v/v) human AB serum (Gibco), 1000 U/ml penicillin (Gibco), 1000 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), and cytokines (10 IU/ml rhIL-2 and 10 ng/ml

rhIL-7) for 7 days in an atmosphere of 5% CO_2 in air at 37 °C. In one set of experiments, autologous PBMC were pulsed with the antigen and used as APC analogous to DC.

2.7. Generation of $p53_{22-36}$ peptide-specific $CD4^+$ T cells

PBMC were isolated by Ficoll–Hypaque density gradient centrifugation of venous blood obtained from an HLA-DRB1 * 0401 healthy donor. Autologous DC were generated from the PBMC as described above and pulsed with the synthetic peptide. The $CD4^+$ peptide-specific T cell line was generated in IVS cultures, using the peptide-pulsed DC as stimulators and characterized as previously reported (Chikamatsu et al., 2001).

2.8. IL-5 ELISPOT assay

ELISPOT assay was performed in wells of 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA), using anti-human IL-5 (PharMingen; catalog no.18051D) as a capture mAb and biotinylated anti-human IL-5 (PharMingen; catalog no.18522D) as detection mAb. Wells were coated with 100 μl of capture mAb solution (10 μg/ml in 1 × PBS; pH 7.4), and the plates were incubated overnight at 4 °C. On the following day, wells were washed 4 × with phosphate buffered saline (PBS; Gibco/Life Technologies). Next, 200-μl aliquots of RPMI medium supplemented with 10% (v/v) human serum were added to each well for 2 h at 37 °C to block non-specific binding. Responder (L) and APC cells resuspended in AIM-V medium were then added (the ratio of L:APC was 10:1), and the assay was incubated for 48 h. Next, the plates were vigorously washed 6 × with the solution of 0.05% Tween 20-PBS, and the biotinylated detection anti-IL-5 Ab was added at 1 μg/ml. The plates were again incubated at 37 °C for 4 h. The washing steps were repeated, and after a 1-h incubation at room temperature with the avidin–peroxidase complex reagent (Vectastain Elite Standard ABC-Kit; Vector Laboratories, Burlingame, CA), the plates were washed 3 × with PBS/0.05% Tween and then again 3 × with PBS alone. Following addition of the substrate, the color reaction was stopped after 5 min by extensive washing. The spots were counted by computer-assisted image analysis (Zeiss ELISPOT 4.14.3; Zeiss, Jena, Germany). The frequency of positive (IL-5-producing) cells per total number of cells plated per well was calculated after the number of spots in control wells had been subtracted from that in experimental wells. The control wells contained non-pulsed APC and responding lymphocytes. Various experiments were performed in order to establish the IL-5 ELISPOT assay as follows.

(a) *Cross-titration of anti-IL-5 Abs*. In order to determine the optimal concentrations of the capture and detection mAbs, normal human PBMC activated with PHA (5 μg/ml) were plated in triplicate wells of 96-well plates coated with various dilutions of the capture Ab. After 48 h of incubation, supernatants were removed, and after extensive washing, the detection Ab was added at various dilutions. These checkerboard titrations were performed in at least three independent experiments.

(b) *Use of different APC*. As APC, either autologous PBMC, monocyte-derived DC or T2 cells, as appropriate, were pulsed with the stimulating antigens and added to each well at the 10:1 ratio of L:APC. The T2.DR4 cell line, used in some experiments, was generated through transfection of HLA-DRB1 * 0401 cDNA into T2 cells (Bertoletti et al., 1997). The plates were incubated for 24 or 48 h at 37 °C.

(c) *Evaluation of different substrates.* To optimize the assay, two different substrates 3,3′ , 5,5′ -tetramethylbenzidine (TMB; Vector laboratories, Burlingame, CA) or of aminoethylcarbazole (AEC) staining solution (Sigma), were evaluated in a series of triplicate experiments performed in parallel with both substrates.

(d) *Evaluation of the assay.* Inter- and intra-assay reproducibility of the ELISPOT assay for IL-5 were determined using freshly harvested PBMC obtained from six normal volunteers on different days and stimulated with PHA. To establish linearity of the response curve, PBMC were re-suspended in AIM-V medium and added at various numbers, ranging from 12,500 to 10^5 per well in triplicate determinations.

2.9. Selection of the assay format using VLP-L1 as antigen

Four different ELISPOT assay formats were evaluated in this study with the goal of establishing a

Table 1
Titration of the capture and detection Abs for use in IL-5 ELISPOT assays[a]

Detection Ab (μg/ml)	Spots counted/10^5 PBMC plated per well				
	Capture Ab (μg/ml)				
	1	2	5	10	15
1	5 ± 4	24 ± 5	190 ± 20	236 ± 25	165 ± 12
2	4 ± 2	15 ± 2	200 ± 34	197 ± 43	142 ± 18
5	6 ± 3	16 ± 1	157 ± 14	209 ± 27	185 ± 21
10	9 ± 1	15 ± 6	112 ± 3	190 ± 7	173 ± 9

[a] Checkerboard titrations were performed to determine the optimal number (± SD) of spots per well in an ELISPOT assay for IL-5 production. PBMC of one normal donor, used as responders, were titrated into wells (10^5/well) and stimulated with PHA (5 μg/ml) for 48 h. The capture and detection Abs described in Materials and methods were used at various concentrations, as indicated. The combination of 10 μg/ml of capture Ab and of 1μg/ml of detection Ab gave a significantly higher number of spots ($p < 0.05$ by the permutation test) than the 5 and 2 μg/ml of the Abs. The results are from a representative experiment of three performed with PBMC obtained from different donors.

reproducible assay, which could be easily applied to the immune monitoring of T cell responses to VLP-L1. These assay formats were as follows.

(a) A direct assay, in which PBMC obtained from normal individuals or volunteers vaccinated with VLP-L1 were used as APC. The PBMC were pulsed with VLP-L1 for 18 h and then added to wells of ELISPOT plates containing 10^5 autologous PBMC used as responding cells (R) to give the ratio of 10R:1APC.

(b) A direct assay, in which immature DC were pulsed with VLP-L1 for 4 h, matured in cytokines and then combined with PBMC at the ratio of 10 PBMC:1DC in ELISPLOT plates.

(c) An assay with IVS, in which PBMC pulsed with VLP-L1 were cultured for 7 days and then used as responders in 48-h ELISPOT assay with autologous PBMC pulsed with VLP-L1 for 18 h at the ratio of 10R:1APC.

(d) An assay with IVS in which DC pulsed with VLP-L1 for 48 h and matured with cytokines overnight were used as APC during a week-long culture as well as in 48-h ELISPOT assay.

2.10. Statistical analysis

In ELISPOT assays, numbers of spots in triplicate wells were compared vs. controls using the permutation test. Differences in frequencies of IL-5 producing cells between normal or immunized donors or assays performed under different conditions were evaluated using the Wilcoxon test. Whenever applicable, Student's *t*-test was also used. Changes in the frequency of IL-5 producing cells in individual donors were defined as exceeding a 95% confidence bound calculated from repeated measurements of normal controls, who were assumed to represent normal biologic variability between measurements. Differences with a p value < 0.05 were considered significant. Inter- and intra-assay reliability was evaluated by calculating the intra-class correlation coefficient, the within-subject coefficient of variation and the standard error of measurement. For all experiments, the 95% confidence interval for a new frequency measurement of one responder per 1000 cells was calculated based on the standard error of measurement.

Table 2
Comparison of color development using AEC or TMB substrates[a]

		Spots counted/10^5 PBMC									Mean
		Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	
Donor 1	AEC	53	84		58	51	86	79	62		68
	TMB	190	188	172	174	166	164	213	181	170	180
Donor 2	AEC	113	156	158	148	120	118	109	140	130	132
	TMB	159	238	223	247	242	213	214	186	201	214

[a] Two different substrates were compared for color development in IL-5 ELISPOT. PBMC of two normal donors, used as responders, were titrated into ELISPOT wells (10^5/well) and stimulated with PHA (5 μg/ml) for 48 h. Next, the capture Ab and then the substrate for color development were added. The data are numbers of spots counted/well. Data are from one representative experiment of three performed.

3. Results

3.1. Assay development

3.1.1. Cross-titration of anti-IL-5 Abs

To establish the ELISPOT assay for IL-5, we evaluated two monoclonal antibodies (mAbs) directed against different determinants of human IL-5. As shown in Table 1, the optimal Ab dilutions were determined to be 10 μg/ml for the capture Ab and 1 μg/ml for detection Ab. The lots of the Abs titrated as described above were reserved and purchased in bulk to ensure their availability for monitoring.

Table 3
IL-5 ELISPOT assay: fresh vs. cryopreserved responder lymphocyte[a]

	Assay date (month/day/year)	Frequency of IL-5-producing cells
Donor 1	02/05/01	1/1299
	03/11/01	1/1075
	04/04/01	1/1111
	03/19/01[b]	1/1064
	04/09/01[b]	1/990
Donor 2	01/23/01	1/532
	02/05/01	1/526
	03/12/01	1/476
	03/19/01[b]	1/617
	04/09/01[b]	1/571
Donor 3	01/23/01	1/500
	02/06/01	1/568
	03/12/01	1/575
	03/19/01[b]	1/562
	04/09/01[b]	1/549
Donor 4	02/05/01	1/348
	03/12/01	1/320
	04/09/01	1/339
	03/19/01[b]	1/500
	04/09/01[b]	1/350
Donor 5	02/06/01	1/383
	03/12/01	1/397
	04/25/01	1/373
	03/19/01[b]	1/350
	04/09/01[b]	1/361
Donor 6	02/08/01	1/1020
	03/19/01	1/1063
	04/09/01	1/1000
	03/19/01[b]	1/1020
	04/09/01[b]	1/962

[a] ELISPOT assays were performed with either fresh or cryopreserved PBMC obtained from six normal donors. Cells were stimulated with PHA (5 μg/ml) in direct ELISPOT assays performed as described in Materials and methods. Assays were performed on different days with PBMC of the same donors.

[b] Cryopreserved cells of each donor were thawed at two different time points and tested in ELISPOT assays. The difference in the frequency of IL-5-producing cells between fresh and cryopreserved PBMC was not significant at $p = 0.4936$.

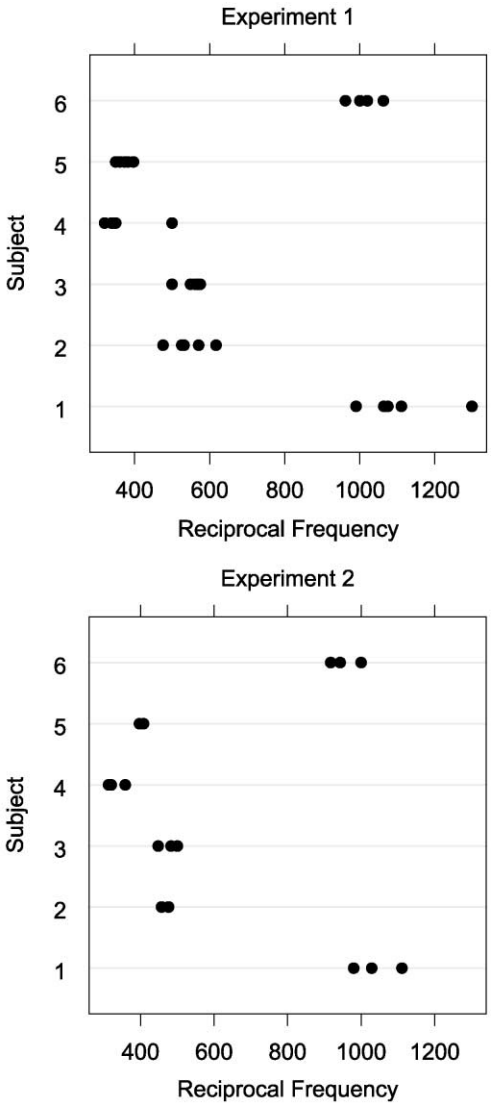

Fig. 1. To measure inter-assay variability, PBMC were obtained from six normal donors and tested in IL-5 ELISPOT assays after PHA stimulation (see Materials and methods) in assays performed on different days (Experiment 1). To measure intra-assay variability, assays were performed on the same day, but were plated in different plates (Experiment 2).

3.1.2. Color development

Two different substrates, AEC and TMB, were compared for color development as shown in Table 2. PBMC obtained from two normal donors were stimulated by PHA, and the assay was developed using two different substrates. TMB gave a higher number of spots, with the ratio of TMB/AEC ranging from 1.6 to 2.6. This increase in spot numbers reflected the clear-cut definition of the TMB spots. While red color spots obtained with the AEC substrate were typically pale and difficult to distinguish from the background, those observed when TMB was used as the substrate had a strong blue color and were well defined. Thus, TMB which allowed for more accurate counting of spots by image analysis, was selected for subsequent experiments.

3.1.3. Length of the assay

To determine the optimal time for spot development, PBMC obtained from six normal donors were stimulated with PHA (5 μg/ml) in ELISPOT plates for various periods of time, ranging from 24 to 72 h. The highest number of spots was consistently observed at 48 h (data not shown). When PBMC of the individuals immunized with the HPV16-L1 vaccine were stimulated with VLP in ELISPOT assays, the need for the 48-h incubation period to obtain the optimal response was confirmed (data not shown).

3.1.4. Fresh vs. cryopreserved cells

Comparisons of fresh with cryopreserved PBMC obtained from six different donors were performed to determine whether this ELISPOT assay could be reliably used with banked lymphocytes. No significant difference ($p = 0.4936$) was observed in the frequency of IL-5-producing cells in fresh vs. cryopreserved cells stimulated with PHA (Table 3).

3.1.5. Reproducibility of IL-5 ELISPOT assays

To evaluate the intra- as well as inter-assay variability of the assay, PBMC obtained from six normal donors were tested using PHA as a non-specific T-cell stimulator. For evaluating inter-assay variability, the same donor's cells ($n = 6$ donors) were tested in assays performed on different days (at least three separate days, as shown in Experiment 1, Fig. 1). To measure intra-assay variability, assays performed on the same day, but plated in different plates, were compared (Experiment 2, Fig. 1). From the data shown in Fig. 1, we calculated that the within-subject coefficient of variation (CV = within-subject SD/overall mean) was 1.6% for inter-assay and 0.8% for intra-assay variability. The intra-class correlation coefficient (i.e., correlation of assay results within the same individual) was 0.956 for assays performed on different days and 0.968 for assay performed on the same day. These results indicate that the assay reliability is excellent.

Table 4
Limiting dilution of samples and inter-assay reproducibility in ELISPOT[a]

Day	Cells/well	Spot count					Spots/10^6 cells	Frequency
		Well 1	Well 2	Well 3	Mean	SD		
Day A	100,000	219	222	230	224	6	2237	1/447
	50,000	94	96	92	94	2	1880	1/531
	25,000	46	42	49	46	4	1827	1/547
	12,500	26	19	23	23	4	1813	1/551
Day B	100,000	233	230	255	239	14	2393	1/419
	50,000	71	82	72	75	6	1500	1/667
	25,000	37	43	31	37	6	1480	1/676
	12,500	16	25	18	20	5	1568	1/638
Day C	100,000	234	194	235	221	23	2210	1/452
	50,000	102	79	91	91	12	1993	1/502
	25,000	39	44	33	39	6	1546	1/647
	12,500	26	22	25	24	2	1946	1/514

[a] ELISPOT assays were performed on different days (A, B, C,) using freshly isolated PBMC of the same normal individual. The PBMC were titrated into wells of ELISPOT plates (10^5 to 12,500/well) in triplicate, and activated with PHA (5 μg/ml). Triplicate control wells containing PBMC incubated in medium alone were also plated. Following 48 h of incubation at 37 °C, all control wells were negative (no spots). The presented data are numbers of spots/well.

Serial dilutions of the samples were also performed (Table 4) in order to determine the linearity and limiting dilution of the response. The samples plated in triplicate were scored for the number of spots at each cell concentration. The final mean numbers of spots calculated for each cell dilution were very close and confirm the excellent reproducibility of the assay.

3.1.6. Antigen concentration

In the initial experiments, we evaluated the concentration of VLP-L1 which would give the best responses in ELISPOT assays, using PBMC of the immunized donors. The VLP-L1 preparation was tested at the concentrations of 0.1, 1.0 and 5.0 μg/ml in both IVS-based and direct ELISPOT assays, and the results indicated that 1.0 μg/ml consistently induced the best responses (i.e., gave the highest number of spots; results not shown). In addition, the number of responding cells plated per well had to be adjusted, depending on the assay format. In direct ELISPOT assays (no IVS), we generally plated 10^5 PBMC or enriched T cells. On the other hand, after 7-day cultures with IVS, 10^4 responder cells/well were plated, especially when PBMC of the immunized donors were tested. With established T cell lines, such as wt p53 peptide-specific $CD4^+$ T cells, we plated 10^3 responder cell/well. In some of the initial control experiments, SF9/baculovirus lysate was used for pulsing of APC. These ELISPOT assays were always negative (data not shown).

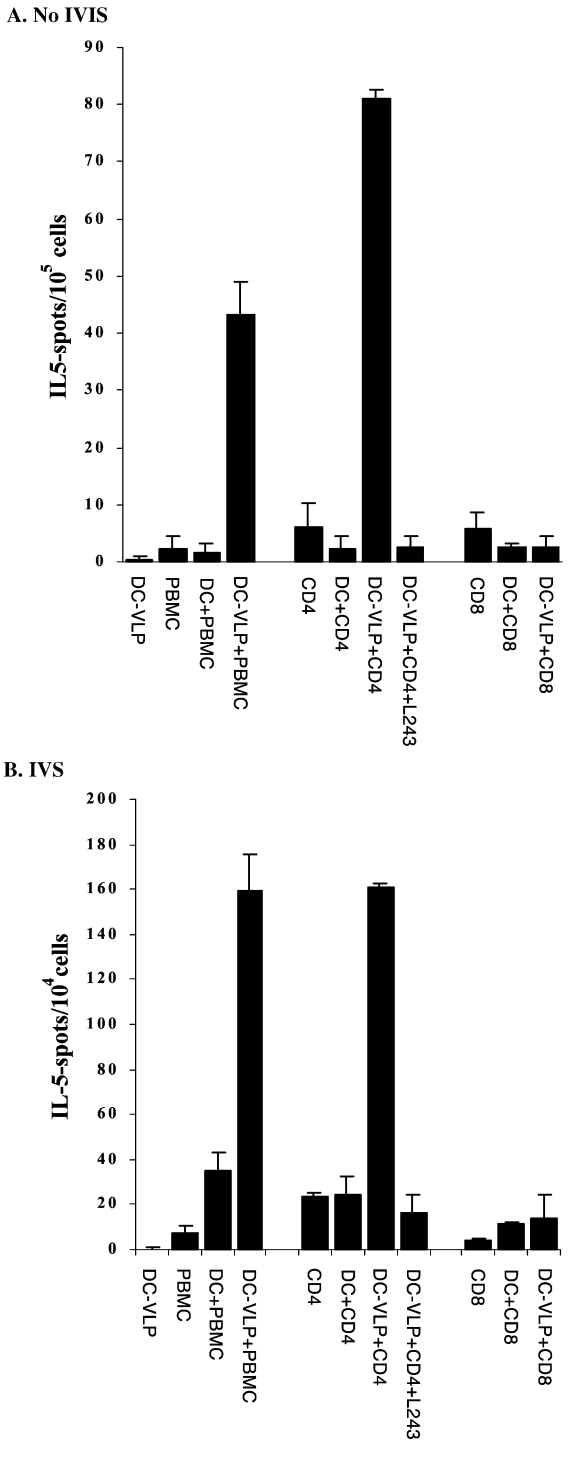

Fig. 2. IL-5 secretion by peripheral blood lymphocytes obtained from the same donor immunized with VLP and stimulated with dendritic cells presenting VLP (DC-VLP). The IL-5 ELISPOT assay was performed without IVS (A) or after one 7-day IVS cycle (B). Either unseparated PBMC or T cells (CD4+ or CD8+) after separation served as responders. T cells generated in IVS co-cultures were enriched in CD4+ and CD8+ T cells by positive selection with magnetic beads prior to ELISPOT assays. For CD4+ T cells, HLA-class-II-restriction was confirmed by adding anti-DR (L243) Ab. Spots were counted by computer-assisted image analysis. The data are numbers of spots for background control and experimental wells. Note that the background values were not subtracted from experimental values. As controls, DC pulsed with VLP; PBMC or CD4+ or CD8+ T cells alone; or unpulsed DC+ PBMC or DC+ CD4+ T cells or DC+ CD8+ T cells were included in the assay. A representative experiment of three performed with autologous DCs and T cells obtained from each of the two donors vaccinated with VLP is shown.

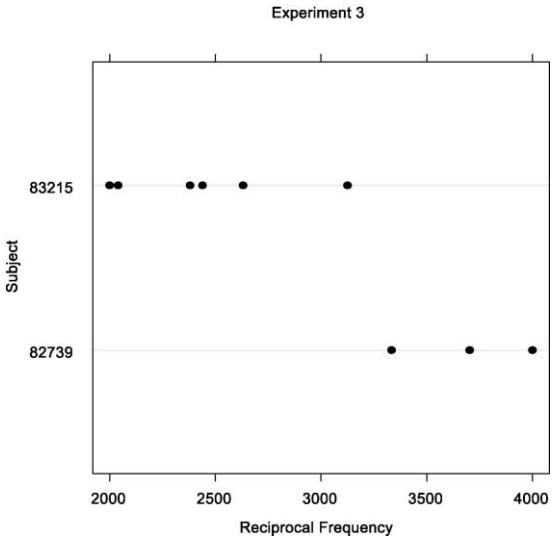

Fig. 3. Reciprocal frequencies of VLP-L1 responding lymphocytes established for two donors immunized with VLP-L1. The cryopreserved PBMC of these donors were thawed and tested on different days in ELISPOT assays for IL-5.

3.2. The format of IL-5 ELISPOT for VLP-L1

PBMC and $CD8^+$ or $CD4^+$ T-cells isolated from PBMC of two volunteers immunized with VLP-L1 vaccine as well as PBMC obtained from seven non-immunized donors were tested using the four ELISPOT assay formats described in Materials and methods. The direct assay (format(a)), in which autologous PBMC were used as APC, was negative in non-immunized as well as immunized donors (data not shown). After IVS with PBMC as APC, the frequency of spots was very high, even when responder PBMC alone (no VLP) were plated as controls, and in view of this high background, it was not possible to evaluate specific responses to VLP. On the other hand, using DC as APC and PBMC of immunized donors as responders, we detected IL-5-secreting T cells with or without IVS (Fig. 2). As expected, the frequency of VLP-1-specific T cells was higher after IVS than in a direct ELISPOT assay (Fig. 2). For example, after subtracting the background spots (i.e., DC + CD4) from the experimental spot numbers (i.e., DC − VLP + CD4), the frequency of IL-5-secreting CD4+ T cells was about 1/73 ($137/10^4$ T cells plated) with IVS and 1/1266 ($79/10^5$ T cells plated) without IVS. In non-immunized donors, no spots were detected using DC as APC in the assays without IVS. However, after 7 or 14 days of IVS with DC pulsed with VLP, responses were observed in 5/7 specimens tested. After IVS, the range of frequencies for IL-5-secreting PBMC of non-immunized donors was 1/1852 to 1/5882 which was lower than that observed for donors immunized with VLP-L1 (1/81).

Based on these results, we selected an ELISPOT assay format that called for the use of DC pulsed with VLP as stimulators and autologous uncultured PBMC (no IVS) as responders. We determined that this assay would be sensitive enough to detect IL-5-secreting cells present in the circulation at the frequency exceeding 1/5000. In the two volunteers immunized with VLP-L1, the frequency of IL-5-secreting cells was 1/2436 and 1/3678. This assay does not require IVS and is feasible for patient monitoring, provided a sufficient number of cryopreserved PBMC is available for DC generation.

After selecting the optimal assay format, we next determined the assay reproducibility by repetitive testing of PBMC obtained from two donors immunized with VLP in ELISPOT assays performed on different days. As shown in Fig. 3, the within-subject coefficient of variation for the two donors tested on six and three different days, respectively, was 1.9% with the intra-assay correlation coefficient of 0.77 ± 0.065 (SEM). Thus, the 95% confidence interval for a new frequency measurement of 1/1000 was estimated to be 1/746 to 1/1340, using this format of the ELISPOT assay for IL-5.

Table 5
ELISPOT assays for IL-5 production by individual T cells responding to the wt $p53_{22-36}$ peptide[a]

Assay conditions	Frequency of IL-5-producing T cells
CD4 (spontaneous release)	1/217
CD4+ T2 DR4+ cells	1/370
CD4+ T2 DR4+ cells + p53 peptide	1/33
CD4+ T2 DR4+ cells + irrelevant p53	1/400

[a] The CD4+ T cell line specific for the wt $p53_{22-36}$ peptide was established in our laboratory (Chikamatsu et al., 2001). For the 48-h ELISPOT assay, p53 peptide (10 μg/ml) was pulsed on T2DR4 cells which were used as stimulators. The effector/target cell ratio was 1:1 in all experiments.

3.3. The frequency of p53 peptide-specific T helper cells

To further evaluate the performance of the ELISPOT assay in the assessment of tumor antigen-specific IL-5-secreting T cells, we used it to determine the frequency of wt $p53_{22-36}$ peptide-specific T cells in a $CD4^+$ T cell line established in our laboratory (Table 5). The frequency of T cells able to respond to the wt p53 peptide was 1/33 in this line. This result indicates that, in addition to measuring the frequency of VLP-responsive T cells, the IL-5 ELISPOT assay could be used to quantify tumor antigen-specific Th2 cells.

4. Discussion

IL-5 is considered to be a Th2-type cytokine involved in B-cell growth and in antibody synthesis. An IL-5 ELISPOT assay has been previously used for quantitation of proteolipid protein (PLP)-specific T cells in patients with multiple sclerosis (Pelfrey et al., 2000). It has also been used in murine models of infectious or autoimmune diseases to characterize T cell responses to antigens potentially involved in the pathogenesis of these diseases (Taguchi et al., 1990; Medhat et al., 1998; De Franco et al., 1995). The contribution of Th2 cells to anti-tumor immunity has also been demonstrated in murine gene therapy experiments, using Th2 cytokines or by adoptive transfer of tumor-specific Th2 clones (Allione et al., 1994; Shen and Fujimoto, 1996). In both instances, tumor regression and activation of tumor- specific $CD8^+$ cytotoxic T lymphocytes was observed (Allione et al., 1994; Shen and Fujimoto, 1996). Based on studies in murine tumor models, Nishimura et al. (1999) have emphasized that Th1 and Th2 cells play distinct roles in the destruction of tumor. While Th1 cells appeared to be the major effectors of the immune cellular response, Th2 cells colonized the tumor blood vessels, inducing inflammation and tumor necrosis (Nishimura et al., 2000). However, in this and other tumor models as well as infectious disease models, immunological memory was not induced using Th2 cell therapy alone (Nishimura et al., 2000; Tepper et al., 1989), indicating that an effective or complete anti-tumor response requires coordinate activity of both Th1 and Th2 cells.

Traditionally, the presence and titers of neutralizing anti-viral antibodies in serum are monitored to document resistance of an individual to a viral infection. In evaluating responses to viruses, for example to HPV, and specifically to HPV-16 capsids, total IgG and IgG-1 type titers generally reflect the cumulative, lifelong exposure to HPV (Wang et al., 2000). The appearance of IgM or IgA antibodies signifies an ongoing or recent HPV infection. In adult volunteers vaccinated with HPV16-L1 VLP, a strong IgG1 response was noted, which was approximately 40-fold higher than that observed after a natural infection (Schiller and Lowy, 1996). This Ig isotype reflects the presence of a Th1-type response to HPV16-L1 VLP. However, transient seropositivity for IgM and also seropositivity for IgA was also observed by ELISA, confirming the generation of a Th2 response. In a recent phase 1 study conducted in healthy adult volunteers who received a recombinant VLP-L1 vaccine prepared from HPV type 11, similar Ab profiles were observed (Evans et al., 2001). In addition, IL-5 and IFN-γ production were evaluated by ELISA and the post-vaccine increases in the levels of these cytokines indicated that robust humoral as well as T-cell responses were generated as a result of vaccination.

While changes in the isotype and levels of anti-HPV antibody titers remain the main aspects of measuring anti-HPV immunity, attempts have also been made to monitor cellular responses to HPV, particularly in the context of developing HPV vaccines (Schiller and Lowy, 1996; Evans et al., 2001). These responses have been elusive and difficult to quantitate, largely due to poor sensitivity and reproducibility of the methods employed. Recent applications of ELISPOT assays for IFN-γ production to quantification of anti-viral responses[1] has allowed for a more precise estimation of the magnitude (i.e., frequency) of virus antigen-specific Th1 cellular responses (Del Prete et al., 1994). However, since HPV-VLP may also be used as a vaccine capable of promoting specific T-cell responses, the need for measuring Th2 reactivity as a co-factor for antibody responses has become apparent. We report here the

[1] Bennouna J., Hildesheim A., Gooding W., Whiteside T. Human pupillomavirus-16 L1 virus-like particles (VLP) presented by dendritic cells elicit Th1 and Th2 responses in humans. Manuscript in preparation, 2001.

development, evaluation and application to monitoring of Th2 responses to VLP of an ELISPOT assay for IL-5. In the past, ELISPOT for IL-5 was compared to ELISA by Tanguay and Killion (1994) and found to be many fold more sensitive. Our choice of IL-5, instead of IL-4 production, for evaluation of Th2-type responses was based on the observations that the former is a more reproducible assay with a high ''signal-to-noise'' ratio (i.e., much lower non-specific background). The use of DC as APC in the assay was carefully scrutinized, as the generation of these cells from relatively small blood samples in patients may represent a time-consuming and logistically demanding assay variable. Our results indicate that this assay format is feasible for use in serial monitoring of patients, as long as approximately 30×10^6 of PBMC are available for the assay. Indeed, our DC-based ELISPOT assays allowed for a reproducible discrimination of the frequency of antigen-specific T-cells in non-immunized donors as well as individuals immunized with antigen.

While we used this assay to illustrate the magnitude of responses to HPV-VLP in non-immunized and HPV-immunized human donors, it is important to stress that the assay is equally applicable to quantitation of responses to other complex antigens, e.g., tumor-specific polypeptide antigens. As an example, we used the IL-5 ELISPOT assay to measure the frequency of CD4+ T cells specific for the wt $p53_{22-36}$ peptide presented on $DR4^+$ T2 cells in the wt $p53_{22-36}$ peptide-specific $CD4^+$ T-cell bulk line generated and maintained in our laboratory. As humoral responses to p53 can be documented in a subset of patients with cancer (Lubin et al., 1995), we expected that p53-specific CD4+ effector cells may be present in the circulation of such patients. However, using peptide-pulsed DC, we recently succeeded in the generation of wt p53 peptide-specific CD4+ T cells not only from PBMC of DR4+ patients with cancer but also from normal DR4+ donors (Chikamatsu et al., 2001). One such CD4+ bulk cell line able to recognize tumor, contains 3% of such T-cells, which secrete IL-5 and which are HLA-DR4 restricted[2] (Chikamatsu et al., 2001).

In the future, we anticipate that vaccinated patients will be monitored using IFN-γ and IL-5 ELISPOT assays in order to follow Th1 and Th2 response profiles, respectively. A more complete assessment of the immune response may be useful in identifying patients who are most likely to respond to vaccines, in monitoring evolving immunity and potentially in predicting disease reoccurrence in successfully treated individuals.

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